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# Modulation of RANKL and Osteoprotegerin in Adolescents Using Orthodontic Forces

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# Modulation of RANKL and Osteoprotegerin in Adolescents Using Orthodontic Forces

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**MODULATION OF RANKL AND OSTEOPROTEGERIN IN  
ADOLESCENTS USING ORTHODONTIC FORCES**

A Thesis  
Presented for  
The Graduate Studies Council  
The University of Tennessee  
Health Science Center

In Partial Fulfillment  
Of the Requirements for the Degree  
Master of Dental Science  
From The University of Tennessee

By  
Nathan Reed Hamman, D.D.S.  
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## ABSTRACT

Orthodontic tooth movement is mediated by interactions between PDL cells and those of the alveolus. One protein – the receptor activator nuclear factor kappa B ligand (RANKL) – is critical for osteoclastogenesis, and osteoprotegerin (OPG) is a decoy ligand that competitively inhibits RANKL. A higher RANKL/OPG ratio is associated with areas of bone resorption, while a lower ratio occurs in areas of bone deposition and homeostasis. There has been almost no clinical study of RANKL and OPG expression in patients undergoing orthodontic tooth movement.

The purpose of this study was to quantify changes in the levels of RANKL and of OPG in response to orthodontic forces. Untreated adolescents had a calibrated force applied across a left-right pair of maxillary premolars with a transpalatal spring (TPS). RANKL and OPG was measured in gingival crevicular fluid (GCF) sampled serially from the pressure and tension sides of maxillary premolars at 5 time points: before placement of transpalatal spring, 2 days after TPS placement, 5 days after TPS placement, 10 days after TPS placement (TPS was then removed), and 7 days after TPS removal. RANKL and OPG expression was measured by ELISA assay. Expectations were that (1) force would raise RANKL and diminish OPG, (2) force removal would reverse the RANKL-OPG levels, (3) strength and duration of force are associated with RANKL-OPG levels, and (4) responses would exhibit considerable inter-individual variation.

Gingival crevicular fluid (GCF) volume increased significantly after applying force with the nickel-titanium coil spring. The volume remained elevated until the force was removed, and had returned to baseline by one week. Low detectability of RANKL and OPG in the samples, which can be partially attributed to low volumes of GCF, made it impossible to assess the effects of orthodontically induced changes in their concentrations. The levels of these molecules present in the GCF collected, as well as their changes over time, were, more than likely, too small to be measured with current commercially available ELISA assay kits. It is also possible that a large change in RANKL or OPG expression could have occurred after spring placement and before the first sample of GCF was collected (between 0 and 48 hours). Further research is needed to determine what effect variations in individual RANKL and OPG expression have on orthodontic tooth movement.

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## CHAPTER 1. INTRODUCTION

Storey (1972) described bone as a simple tissue consisting of four components: cells, the extracellular matrix of collagen fibers, mucopolysaccharide ground substance, and calcium salts. Although bone is a simple material, it is also dynamic and is undergoing continual remodeling and mineralization. Physiological bone remodeling is aimed at regulating the body's free calcium ion supply, maintaining an adequate blood supply to osteocytes embedded in the bone, and adapting to the mechanical stresses and strains of everyday functions. To accomplish these processes, a balance is maintained between osteoblastic (synthesis) and osteoclastic (resorption) activities. When orthodontic forces are applied to a tooth, they are transmitted into the periodontal ligament (PDL) and adjacent alveolar bone, which causes a shift in osteoblastic/ osteoclastic activity. On the side of the tooth where the fibers of the PDL are stretched, an environment is created where osteoblastic activity predominates and new bone is formed. Areas where the PDL are under compression exhibit increased osteoclastogenesis, leading to bone resorption (Masella and Meister 2006).

The interactions between the cells of the bone and periodontal ligament with the extracellular matrix, as well as cell to cell interactions involved in orthodontic tooth movement (OTM), are complex. The sequence of events that occurs at the tissue and cellular levels during OTM is well understood. However, there is still a lack of comprehensive understanding of the orchestration of biochemical events at the molecular level in response to orthodontic force (Krishnan and Davidovitch 2006). Discoveries in the molecular biology of bone, such as specific regulatory molecules and pathways, have given the specialty of orthodontics a new appreciation of the complexity of the events that must occur for OTM to take place. In recent years, attention has focused on two osteoblast-derived factors that play key roles in bone growth and remodeling. The receptor activator of the nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) exert counterbalancing regulatory effects on osteoclast differentiation, activation, and survival, and are therefore critical for initiation and maintenance of OTM (Kanzaki *et al.* 2006). The aim of the present study was to measure changes in RANKL and OPG expression in human gingival crevicular fluid, as well as their relative ratio in areas of tension and compression, during application of orthodontic force.

Little is known about the levels of RANKL and OPG in human gingival crevicular fluid (GCF) in people undergoing orthodontic treatment. Most of the studies that have assayed GCF have focused on people with periodontitis, where osteoclastogenesis is up-regulated. Data collected during the present study are

useful in determining the levels of RANKL and OPG in the GCF of adolescents in the absence of (prior to) application of orthodontic force. Kawasaki *et al.* (2006) found that these levels increase with age – at least in their evaluation in a sample of adolescents compared to a sample of adults evaluated cross-sectionally. The present study focused on adolescents between the ages of 12 and 18 years at their initial orthodontic visit, which is the age interval for the great majority of orthodontic patients in the United States. There is particular interest in the interindividual variability of the sample, since differences in RANKL seem to be tied to an individual's risk of external apical root resorption (*e.g.*, Harris *et al.* 1997; Harris 2000).

After application of force of a known magnitude, initial changes in RANKL and OPG expression in GCF were analyzed. Expectation was that RANKL would increase and OPG would drop. Serial sampling of GCF was expected to provide information on whether the sustained tension promotes sustained levels of the molecules or whether the responses are dynamic over time. Force removal was used to test the recovery of the system towards or, even, to the baseline levels during the subsequent week. It also was of interest whether the duration-to-cytokine levels are simply linear or more complex in nature.

## CHAPTER 2. REVIEW OF THE LITERATURE

### Alveolar Response to Orthodontic Force

#### The Pressure-Tension Response

The pressure-tension model has been the foundation of the concept of orthodontic tooth movement for last century. The idea that orthodontic forces are transmitted into the alveolar bone via the collagen fibers of the periodontal ligament (PDL) originated from early histological research about tooth movement (Sandstedt 1904; Oppenheim 1911; Schwarz 1932). This was a rational hypothesis since, when viewed histologically, a tooth undergoing movement exhibits a compressed PDL side coupled with osteoclastic activity, and a stretched PDL side where new bone is being produced by osteoblasts. However, the classic histological representation of tooth movement overemphasized the role of collagen in tooth support, and it did not take into account the viscoelastic gel properties of the proteoglycan ground substance combined with the cells and blood vessels of the PDL (Meikle 2006). Baumrind (1969) suggested that the PDL behaves like a continuous hydrostatic system, and, based on Pascal's law, concluded that orthodontic force is distributed equally to all regions of the PDL. Therefore, tension could not be applied directly to the alveolar bone by fibers of the PDL. While evidence from tooth movement studies suggests that differential pressures can be created in the periodontium (Meikle 2006), Baumrind's hypothesis that negligible tension is transmitted from PDL fibers to alveolar bone in response to force application has been supported. A study conducted by Heller and Nanda (1979), in which collagen fibers of rat PDL were disrupted by a lathyritic agent, resulted in normal bone remodeling in response to orthodontic force. This suggests that bone formation is not directly stimulated by tension from collagen fibers of the PDL.

Although osteoclastic and osteoblastic activities are not directly stimulated by forces transmitted from the PDL into the alveolar bone, changes in the PDL in response to orthodontic force have an important effect on initiation of bone remodeling. Mechanical strain on the cells of the PDL activates multiple cell signaling pathways that result in osteoclast and osteoblast recruitment and activation (Meikle 2006).

Krishnan and Davidovitch (2006) outlined a model that provides an overview of the pathway of tooth movement. Application of orthodontic force alters PDL fluid pressure and strain gradually develops in the cells and extracellular matrix. The first response of osteoblasts to strain is an increase of

intracellular free  $\text{Ca}^{+}$ , leading to release of prostaglandins that up-regulate osteoclastic bone resorption and osteoblastic bone formation. Mechanical strain is also transmitted directly into the nucleus of PDL cells through their cytoskeleton, leading to transcription of specific genes that regulate tooth movement. Sensory nerve fibers of the PDL are also affected by mechanical strain, leading to a biochemical response. Pain-sensitive nerve terminals of the PDL are distorted, triggering a release of stored substance P, a vasoactive neuropeptide that binds to osteoblast cellular receptors and interacts with local endothelial cells. Circulating leukocytes can then adhere to the activated endothelial cells, and the leukocytes migrate into the extravascular space where they synthesize and release signal molecules. This cascade of events leads to osteoblastic bone formation 40 to 48 hours post-force application (Roberts, Huja and Roberts 2004), and peak osteoclastic activity after about 50 hours (Roberts and Ferguson 1995).

### The Bone-Bending Theory

The bone-bending theory gained popularity in the late 1960s and early 1970s with the experiments and Baumrind (1969) and Grimm (1972). Their studies determined that when an orthodontic force of sufficient magnitude is applied to a tooth, the force is disseminated to all of the tissues in the area including the alveolar bone, the PDL, and the tooth. Baumrind (1969) observed that after force application, the PDL on the pressure side of the tooth was compressed only one-tenth the width of crown displacement. This suggested that alveolar bone is more elastic and more easily deformed than tooth or PDL. The side of the tooth in which the PDL fibers are stretched exhibits an adjacent lamina dura that is deformed towards a concave configuration. Therefore, although the PDL fibers are under tension, the alveolar bone is under compression. In contrast, the PDL of the pressure side of the tooth are under compression, but the adjacent alveolar bone is deformed towards a convex surface that is under tension (Krishnan and Davidovitch 2006). This is in agreement with the current orthopedic principle that areas of compression exhibit bone formation, and areas where bone is under tension experience resorption (Melsen 1999).

Areas of bone deformation also exhibit stress-generated electric potentials. Concave surfaces are associated with electronegativity, and convex surfaces are associated with electropositivity (Bassett and Becker, 1962). This observation lead Gillooly *et al.* (1968) and Zengo *et al.* (1973) to propose that generation of electric potentials are responsible for regulating bone formation and resorption during orthodontic tooth movement. However, this hypothesis is contrary to biological observations. For example, electric potentials are produced not only in

living bone, but dead bone as well. Also, an increased understanding of cell-cell and cell-matrix interactions, has led to the conclusion that stress-generated electric potentials are only a by-product of bone deformation and do not regulate bone remodeling (Meikle 2006).

### Effects of Microcracks on Bone Formation

When pressure inducing plastic deformation is applied to bone, “slip planes” or microcracks are produced (Storey 1972). These microcracks were first observed by Frost (1960), who proposed that they lead to localized areas of necrosis that may induce bone remodeling. A study by Verna *et al.* (2004) examined the density of microcracks formed in alveolar bone due to orthodontic loading on lower molars of three-month-old male Danish land-race pigs. The treated molar’s alveolar bone exhibited significantly more microcracks than the control molar at the beginning of orthodontic load application. Bone on the pressure side of the tooth also exhibited significantly more microcracks than the tension side. These cracks were observed in connection with areas of active resorption. Verna *et al.* (2004) described the resorption associated with microdamage as “targeted remodeling as opposed to ‘random’ remodeling that could serve other functions, such as calcium homeostasis.” These findings suggest that microcracks produced by orthodontic force may be an initial stimulus leading to cellular transformation, activation, and ultimately, remodeling of bone.

### Duration and Magnitude of Force

When applying orthodontic force to the dentition, the goal is to produce the most efficient movement possible while avoiding negative consequences. There have been many studies aimed at determining the optimum duration and magnitude of force that should be used to achieve this goal. Sandstedt’s (1904) early histological studies demonstrated that, on the tension side of the tooth, bone is deposited after both heavy and light forces. The pressure side of the tooth responds differently depending on the amount of force. With light forces, direct resorption of the adjacent alveolar bone is observed. However, with heavy force, the periodontal tissues are compressed and blood flow is disrupted. This produces cell-free areas that Sandstedt termed hyalinization. According to Reitan and Rygh (1994), formation of a hyalinized area of about one to two millimeters in thickness is almost unavoidable in the initial period of tooth movement. When areas of hyalinization are formed, bone resorption is slowed considerably because osteoclasts must be recruited from neighboring marrow spaces to remove the necrotic (cell free, hyalinized) tissue. This resorption takes

place in a manner that Sandstedt described as undermining resorption, and it can take from two to four weeks or longer depending on root surface area (Reitan 1964). After the hyalinized tissue adjacent to the tooth has been removed, tooth attachment is reestablished. The tissue is membranous and rich in cells, and the PDL space is considerably widened during the following period of tooth movement. Osteoclasts resorb bone over a much wider area, and as long as the force applied to the tooth remains within physiological limits, direct bone resorption will continue to occur (Reitan and Rygh 1994). Theoretically, if an optimal force is applied to a tooth, bone resorption on the pressure side, and therefore tooth movement, will continue at a constant rate without a lag phase (Kohno *et al.* 2002).

Schwarz (1932) introduced the concept of optimal force to the orthodontic community. He defined it (1932:350) as the force “not greater than the pressure in the blood capillaries” (20 to 26 grams for 1 sq. cm. surface). He stated that tooth movement would not occur with forces below this level, and forces above the optimal level would lead to areas of undermining resorption and tissue necrosis due to occlusion of the blood vessels. Gianelly (1969) also demonstrated that bone resorption depends on maintaining circulation to the periodontal tissues. Recently, Noda *et al.* (2007) described use of a ratchet bracket that resulted in rapid and pain-free tooth movement by maintaining one-third of the PDL width, which prevented obstruction of blood flow. Storey and Smith (1952) suggested a continuous force in the range of 150 to 250 gm based on clinical observations in which canines rapidly moved into premolar extraction sites after application of forces in this range. Reitan (1964), on the other hand, recommended a force of 50 gm based on histological studies in which he observed that this amount of force resulted in little root resorption and direct bone resorption on the pressure side of premolars after as little as 15 days. Over the years there have been varied opinions on optimal force levels. Ren *et al.* (2003) conducted a systematic review of literature pertaining to orthodontic forces, and they determined (2003:86) that “no evidence about the optimal force level in orthodontics could be extracted from the literature.” This is thought to be due to the large inter-individual (phenotype-mediated) variability in response to orthodontic force. Some factors affecting tooth movement are: type of mechanics used, differences in cementum hardness along the root surface (Chutimanutskul *et al.* 2006), variation in individual alveolar bone composition (*e.g.*, cell numbers, degree of mineralization), variation in expression of key regulatory cytokines (*e.g.*, RANKL and OPG), and alveolar bone vascularity per tissue volume (Masella and Meister 2006). When these factors are taken into account, it is understandable how the “optimal force” varies, not only from patient to patient, but from tooth to tooth within individuals.



## Osteoclast Differentiation and Activation

### Osteoclast Function

Osteoclasts are multinucleated giant cells derived from hematopoietic precursors, and they are only present in bone (Suda *et al.* 1999). These cells are directly responsible for the resorption of bone, whether it be resorption observed during normal bone remodeling or in response to pressure resulting from an external force (*e.g.*, orthodontic force). After the resorptive activity of an osteoclast is induced, the cell adheres to the bone matrix and forms a ruffled border which expresses hydrogen ions that rapidly dissolve the mineralized component of the bone. After which, the collagenous bone component is degraded by lysosomal cysteine proteinases and cathepsins secreted from the osteoclast (Sasaki 2003). Active osteoclasts are also characterized by high expression of tartrate resistant acid phosphatase (TRAP) and cathepsin K (Boyle *et al.* 2003). Osteoclastic bone resorption is fundamental to directed (orthodontic) tooth movement because the supporting alveolar bone on the pressure side of the tooth's PDL must be removed for tooth movement to occur (Roberts *et al.* 1981; Oshiro *et al.* 2002). In tandem, stable tooth support depends on bone formation on the stretched side of the PDL.

### Osteoclastogenesis

Osteoclastogenesis is a biochemical process in which bone marrow precursors differentiate into the specialized multinucleated osteoclasts. The hematopoietic precursor cells have traits of the macrophage lineage and are stimulated to differentiate into preosteoclasts by the cytokine M-CSF (macrophage colony stimulating factor). Following initial differentiation, cell-to-cell contact with osteoblasts or stromal cells expressing RANKL will initiate fusing of multiple preosteoclasts to form a polykaryon. Maturation occurs on bone where the fused polykaryon must again be stimulated by cell-to-cell contact to differentiate into an activated osteoclast capable of resorbing mineralized tissue (Boyle *et al.* 2003). An osteoclast will remain active as long as a positive stimulus is present (expression of RANKL by osteoblasts and stromal cells). When down-regulation of RANKL occurs because the stimulus is removed, osteoclasts rapidly experience apoptosis (Suda *et al.* 1999).

## RANKL and OPG in Osteoclast Regulation

Differentiation and cellular activities of osteoblasts and osteoclasts both are regulated by a variety of molecules, including (1) osteotropic hormones, (2) inflammatory mediators, and (3) growth factors. In recent years, understanding of osteoclast differentiation and activation has greatly increased due to analysis of the tumor necrosis factor (TNF) receptor and TNF-like proteins: receptor activator of NF- $\kappa$ B (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) (Boyle *et al.* 2003).

The receptor activator of the nuclear factor kappa B ligand (RANKL) is a member of the TNF ligand family (Lacey *et al.* 1998), and was discovered as a membrane-bound protein that is present on the cell surface of osteoblasts, stromal cells, and other cell types. Since then, a soluble form of the protein (RANKL3) has also been identified (Ikeda *et al.* 2001). RANKL acts on cells through the receptor activator of NF- $\kappa$ B (RANK) which is a membrane-bound member of the TNF receptor family.

Through cell-to-cell signaling, RANKL has a potent effect on osteoclast differentiation from hematopoietic precursor cells and stimulates their bone resorptive activity (Udagawa *et al.* 1999). Binding of RANKL to an osteoclast RANK site results in intracellular expression of various TNF receptor associated factors (TRAF's) (Wise and King 2008). TRAF6 is thought to be one of the most important of these factors due to its function of activating the signaling pathways for NF- $\kappa$ B (Boyle *et al.* 2003). Also, the survival of mature osteoclasts depends on the presence of RANKL (though other factors, such as IL-1, also promote survival); otherwise, the osteoclasts experience apoptosis. Another key regulator of osteoclastogenesis is the colony stimulating factor CSF-1. CSF-1 up-regulates the expression of the RANK gene in osteoclast precursors, and is therefore necessary for osteoclastogenesis (Arai *et al.* 1999).

Osteoprotegerin (OPG), in contrast, is a novel secreted member of the tumor necrosis factor receptor superfamily that inversely (negatively) regulates osteoclastogenesis (Vitovski *et al.* 2007). OPG also is known as osteoclast inhibitory factor, which is a soluble *decoy* receptor only because it lacks a membrane spanning domain (Feige 2007). Binding of OPG to the RANK site of preosteoclasts competitively inhibits the binding of RANKL, terminating their differentiation into mature osteoclasts (Kanzaki *et al.* 2005). When OPG is added to bone marrow cultures, it inhibits the process of osteoclast generation reversibly (Yasuda *et al.* 1998). Feige (2001) relates that, in mice treated with 10 mg/kg of OPG intravenously, all osteoclasts disappeared within 48 hours. Of interest, osteoclasts returned within 7 to 10 days where they again appear in

normal numbers and typical locations in these mice (Lacey *et al.* 1998). A recent study determined that bone resorption modulated by RANKL and OPG not only stimulated osteoclast differentiation, but also affected changes in osteoblast proliferation “suggesting a feedback mechanism from osteoclasts to osteoblasts” (Lin *et al.* 2007:407).

Osteoclast differentiation and function appear to be regulated by a counterbalancing system that has been referred to as the RANKL/OPG regulatory axis (Boyle *et al.* 2003). This system is controlled by regulation of specific gene expression, and serves the purpose of maintaining bone structure and function, as well as meeting the body’s physiological need for ions sequestered in bone. If the RANKL/OPG ratio increases, favoring osteoclast differentiation and activation, bone resorption will be observed. If the RANKL/OPG ratio decreases, osteoclastic activity will be inhibited and bone formation will predominate (Kanzaki *et al.* 2001). Tight control over the body’s RANKL/OPG regulatory axis must be maintained for normal function. For example, when an orthodontic force was applied to the incisors of OPG-deficient mice, the alveolar bone was severely destroyed and partially perforated at two and five days after force application due to unbalanced osteoclastic resorption (Oshiro *et al.* 2002).

### Functions and Dysfunctions of the RANKL/OPG Regulatory Axis

#### Tooth Eruption and Development

Recent research analyzing the various local signaling molecules surrounding a developing tooth bud has shown significant expression of RANK, RANKL, and OPG (Liu *et al.* 2005). At times of increased osteoclastic activity the RANKL/OPG ratio is increased by either down-regulation of OPG by colony stimulating factor one (CSF-1) or by up-regulation of RANKL by a number of osteogenic cytokines such as tumour necrosis factor alpha (TNF- $\alpha$ ), interleukin one alpha (IL-1 $\alpha$ ), transforming growth factor beta one (TGF- $\beta$ 1), and CSF-1. Studies of RANKL knockout mice have shown that absence of this gene leads to lack of tooth eruption, even when given a RANKL transgene, suggesting that the RANKL needed to initiate and sustain alveolar bone resorption is produced by the dental follicle (Liu *et al.* 2005). RANKL is thought to be expressed primarily in the coronal part of the dental follicle, while OPG is thought to predominate in the apical portion, favoring bone formation.

Ohazama *et al.* (2004) also examined OPG, RANK, and RANKL gene expression during tooth development in mice. Gene expression was analyzed in

explant cultures from epithelial thickening to cytodifferentiation stages. They reported that OPG is weakly expressed in the initial thickening of tooth epithelium, as well as in the outer edges of the mesenchyme during the bud stage. RANKL and OPG are both strongly expressed in the internal and external enamel epithelium throughout the cap stage. When RANKL signaling is temporarily inhibited during tooth development by administration of exogenous OPG, adverse affects are observed. Tooth development is delayed, resulting in thin dentin and enamel and narrower pulp tissue. This suggests that the RANKL signaling system plays an integral role in tooth development, and disruption or dysfunction of the RANKL/OPG axis can have serious negative consequences.

### Primary Tooth Root Resorption

Resorption of primary tooth root structure is a physiological process that is requisite for proper eruption and emergence of the permanent successor (Wise and King 2008). Molecularly, this process is similar to bone remodeling, involving many of the same transcription factors and cytokines. Release of RANKL from a tooth's dental sac seems integral to the dissolution of bone occlusal to the permanent tooth and, thus, a rate-limiting factor in tooth eruption (at least during the preemergent phase). Likewise, RANKL from the succedaneous tooth's dental sac activates odontoclasts, leading to root resorption and the subsequent exfoliation of the primary predecessor. This accounts for primary tooth retention when a successor is congenitally absent (Harokopakis-Hajishengallis 2007).

### Bone Metabolism Dysfunction

The RANKL/OPG system is responsible for regulating a wide variety of biological and physiological processes, many with health-care ramifications. Dysfunction resulting from alterations in the RANKL/OPG ratio accounts for a number of abnormalities in bone dynamics. For example, if the ratio of RANKL to OPG is increased (either from an over-expression of RANKL or a deficiency of OPG), osteoclastic resorption of bone will predominate over osteoid deposition by osteoblasts and osteoporosis will develop (Sasaki 2003). It has been suggested that inhibition of RANKL mediated activation of osteoclasts by OPG may be an effective method of treating the symptoms of osteoporosis, and may also lessen the amount of cartilage destruction seen in arthritis (Jones *et al.* 2002). In contrast, mice with a RANKL deficiency exhibit severe osteopetrosis, defects in tooth eruption, and no osteoclasts due to the inability of osteoblasts to initiate osteoclastogenesis through RANKL/RANK signaling (Kong *et al.* 1999). In the disease process of multiple myeloma, RANKL is up-regulated in the bone

marrow microenvironment. It has also been observed that myeloma cells express RANKL and inhibit the expression OPG (Leenheer 2004), leading to devastating osteoporosis. Alterations in the RANKL/OPG ratio are also observed in the pathological process of Paget's disease (Rifkin and Gay 1992). Farther afield, the RANKL-OPG axis has been implicated in the slowed wound healing seen in type II diabetics, chronic heart disease, and rheumatoid arthritis (Jones *et al.* 2002). It is clear that the RANKL/OPG system is responsible for regulating many biological processes, and that preventing or correcting alterations in the system could help alleviate the symptoms of a number of pathological conditions.

### Obtaining RANKL and OPG from Crevicular Fluid

Research in periodontology shows that RANKL and OPG both are present in gingival crevicular fluid (GCF), and they can be readily obtained clinically (Mogi *et al.* 2004; Bostanci *et al.* 2007). Consequently, GCF samples can be collected noninvasively and longitudinally across time in the same subjects. GCF is an exudate usually produced in response to inflammatory mediators triggered by bacterial insult (*e.g.*, periodontitis, gingivitis). Normally, it is not present in large amounts in the sulcus of periodontally healthy individuals (Abbott and Caffesse 1977). However, orthodontic forces produce an environment that can be described as “a continuous sequence of inflammation and repair designed to restore normal tissue continuity and function” (Meikle 2006:236). This *inflammation* is aseptic, as opposed to the inflammation observed in response to periodontal disease, but the reaction involves many of the same cytokines because orthodontic tooth movement results from a combination of both pathologic and physiologic responses (Wise and King 2008). Iwasaki *et al.* (2005) measured changes in IL-1 $\beta$  and IL-1 receptor antagonist (IL-1RA) expression in human crevicular fluid in response to orthodontic tooth movement. Studies show that GCF containing measurable amounts RANKL and OPG can be obtained from the sulcus of teeth subjected to orthodontic forces (Heinrich *et al.* 2005; Kawasaki *et al.* 2006; Nishijima *et al.* 2006).

### Clinical Relevance

The RANKL/OPG balancing system is important in regulating osteoclastogenesis and is therefore critical to orthodontic tooth movement. A better understanding of this system could lead to new clinical techniques in terms of defining optimal forces for tooth movement (Boberts-Harry and Sandy 2004; Meikle 2007). Two studies by Kanzaki *et al.* (2004, 2006) demonstrate that

orthodontic tooth movement in rats can be inhibited or accelerated by injection of OPG or RANKL, respectively, into the animals' periodontal tissue.

External apical root resorption – a negative consequence of orthodontic treatment – can significantly shorten roots due to resorption of cementum and dentin (Krishnan and Davidovitch 2006). Boyle *et al.* (2003) proposed that variations in RANKL and OPG expression play an important role in root resorption. Odontoclasts and cementoblasts share similar regulatory pathways with osteoblasts and osteoclasts (Sasaki 2003; Oka *et al.* 2007), so there is reason to suppose that increased expression of RANKL is associated with severe root resorption (Al-Qawasmi *et al.* 2006). Recent studies show that heavy orthodontic forces leading to root resorption correspond to elevated RANKL expression in rats (Low *et al.* 2005), and that compressed PDL cells extracted from patients exhibiting severe root resorption expressed significantly more RANKL and significantly less OPG compared to patients exhibiting normal root resorption (Yamaguchi *et al.* 2006). It has been suggested that up to 90% of individual variation in observed root resorption can be attributed to types of mechanics (*e.g.*, intrusion, lingual root torque) (Parker and Harris 1998), but individual variation in RANKL/OPG expression may also result in variations in observed clinical root resorption. Moreover, individual responses to specific vectors (*e.g.*, intrusion, lingual root torque) are themselves modulated by the person's genotype (Hartsfield, Everett and Al-Qawasmi 2004).

## CHAPTER 3. MATERIALS AND METHODS

### Patient Selection

Nothing seems to be known about sexual dimorphism or racial variation in the levels of RANKL or OPG as assayed from human gingival crevicular fluid. To limit potential sources of variation, all subjects (1) were either American whites of European extraction or African Americans (Table 1), (2) were healthy as regards untreated dental caries and periodontal involvement, and (3) between the ages of 12 and 18 years.

For completeness, we also note these 5 exclusionary criteria: Subjects did not (1) have a history of systemic disease; (2) have prior treatment for periodontal disease or active periodontal disease; (3) have acute gingival inflammation; (4) have trauma from occlusion; and (5) had no current use of NSAIDs. This latter restriction is based on recent work that has discovered a reduction of osteoclastic activity in rats after ingestion of Ibuprofen and Aspirin. These NSAIDs inhibit the production of prostaglandins that are responsible for upregulating osteoclastic activity, and consequentially they reduce clinical tooth movement (Arias and Marquez-Orozco 2006). In addition, Kanzaki *et al.* (2002) demonstrated that production of RANKL by cells of the periodontal ligament is up-regulated by prostaglandin E2.

There are insufficient data in the literature to use power analysis to gauge needed sample sizes to detect a sex difference. Instead, rather arbitrary sample sizes of 24 boys and 24 girls were enrolled in the study (total = 48). These sample sizes exceed the number of experimental animals used in recent, relevant dental studies (*e.g.*, Nishijima 2006), and they were within the limits of the cost, time, and effort that can be expended for this pilot assessment.

Prospective subjects were selected from the adolescent pool of patients that presented for screening appointments for orthodontic treatment at the University of Tennessee Health Science Center orthodontics department between January of 2008 and August of 2009.

Table 1. Subject demographics

Sex	n	Caucasian	African American
Male	24	18	6
Female	24	12	12

### Gingival Crevicular Fluid Sampling Protocol

GCF was sampled separately (1) from each tooth and (2) separately from the pressure and the tension surfaces. Samples were stored at -80° C for later processing. Samples were collected at the following time points:

- 1) At baseline, prior to placement of any orthodontic appliance (T1). The TPS was placed after sampling (Figure 1). A spring gauge was used to measure the initial tensional load of the TPS (in the range of 150 to 250 gm) following the protocol of Parris *et al.* (1989).
- 2) Two days (48 hrs) after TPS placement (T2). Tension of the TPS was re-set to T1 value if needed.
- 3) Five days (120 hrs) after TPS placement (T3). Tension of the TPS was re-set to T1 value if needed.
- 4) Ten days after TPS placement (T4). The spring was then removed after obtaining the 4th sample.
- 5) Seven days after TPS removal, which is 17 days after baseline sample (T5). Orthodontic treatment (spacers, bands, brackets) was not initiated until the end of this experimental period.

### Sampling and Processing Procedure

- 1) All clinically detectable supragingival plaque was removed from maxillary premolars without touching the gingiva to prevent plaque contamination.
- 2) Sample sites were isolated with cotton rolls and air dried to prevent saliva contamination.
- 3) A paper strip was inserted 1 mm into gingival sulcus on pressure and tension sides of premolars and left for 30 seconds (Figure 2).
- 4) Any strips contaminated with blood were discarded.
- 5) The volume of GCF collected was measured using a Periotron®.
- 6) The paper strips were combined (2 from pressure sides and 2 from tension sides) together into 1.5 mL centrifuge tube containing 250 µL phosphate buffered saline (PBS, pH 7.2) and a protease inhibitor cocktail (Thermo Fisher Scientific Inc.).
- 7) The samples were stored at -80° C for later processing.
- 8) After thawing, the tubes were centrifuged at 2,000X g for 1 minute at 4° C.
- 9) The samples were then processed with enzyme-linked immunosorbent assays (ELISA) kits for RANKL and OPG analysis.





Figure 1. Occlusal and lateral view of transpalatal nickel-titanium spring.

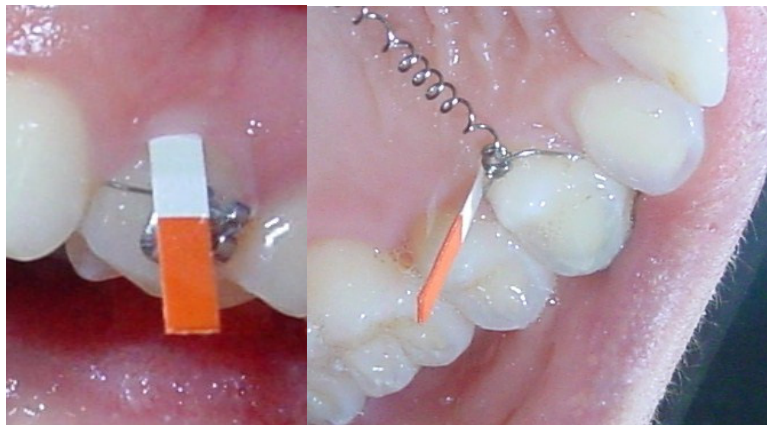


Figure 2. Insertion of perio paper 1 mm into buccal and palatal sulcus.

### Statistical Analysis

The primary question, statistically, was whether the level of RANKL and/or OPG changes within individuals over time given the protocol of (1) inducing mechanical stress on the PDL and adjacent bone and then (2) removing the stress and testing for “recovery” towards baseline levels. Prior studies suggest that there is considerable inter-individual variability and that the distributions may not be normal because of outliers.

Our original intention was to use repeated-measures statics to evaluate changes in expression of RANKL and OPG across time and between pressure and tension sides of the tooth. However, the majority of the samples yielded no usable data, making this impossible. Therefore, factorial models were used although paired differences between sides were tested when possible. The data that was obtained was highly variable (mostly small values, but a few very large values), so non-parametric statistics were used due to the non-normality (*e.g.*, Siegal and Castellan 1988).

Patient’s age, sex, and race were evaluated as covariates, but we did not expect these factors to contribute significantly to the observed variance. It was wholly unknown whether force of the TPS (in the range of 150 to 250 gm) would provide a discernible dose-response effect; we were unaware of any study that had tested for this.

## CHAPTER 4. RESULTS

Orthodontic tooth movement through bone, via resorption by osteoclasts and deposition by osteoblasts, is a complex process initiated by the application of an external force. The histological changes that result from orthodontic forces are well understood (Sandstedt 1904; Oppenheim 1911; Schwarz 1932; Reitan 1967). However, events occurring at the molecular level at the initiation of and during orthodontic tooth movement are, for the most part, unknown. The recent discoveries of the nuclear factor kappa B ligand (RANKL) and its antagonist osteoprotegerin (OPG) have resulted in an increased understanding of the cell signaling and feedback pathways involved in osteoclast/osteoblast activation and function. The aim of the present study was to observe changes in the expression of these two key molecular regulators, specifically in gingival crevicular fluid (GCF), in response to orthodontic force. The results regarding the changes in expression of RANKL and OPG observed over five examinations are discussed below.

The five examinations, with a range of 18 days, are coded in the graphs and tables as day zero (T\_00), day 2 (T\_02), day 5 (T\_05), day 10 (T\_10), and day 17 (T\_17).

### Bioassay Sensitivity

A hindrance to the assessment of the effects of orthodontically induced changes in the concentrations of RANKL and of OPG is the large number of samples in which the levels of these molecules were beneath the level of the technology to detect them. That is, the bulk of samples produced no data because the assays produced no detectable values. These negative results are necessarily listed as missing data. To make the situation clear, the following graphics depict the percentage of unusable data (Figures 3 through 6).

Consequently, statistical analysis is limited, and original intention of using a repeated-measures statistics to monitor levels (A) across time and (B) between lingual and buccal sides of the tooth is not possible. Instead, factorial models are used, though paired differences between sides were tested when possible.

In addition, since the data are so positively skewed (*i.e.*, a few very large values), non-parametric statistics were relied on to deal with the non-normality (*e.g.*, Siegal and Castellan 1988).

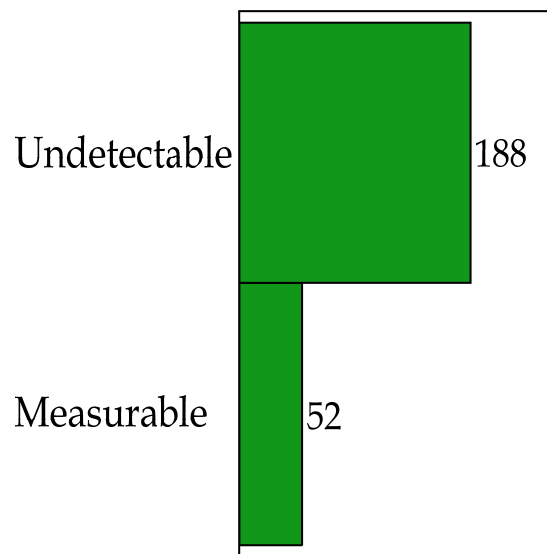


Figure 3. The concentration of RANKL measurable from the lingual side of the premolars.

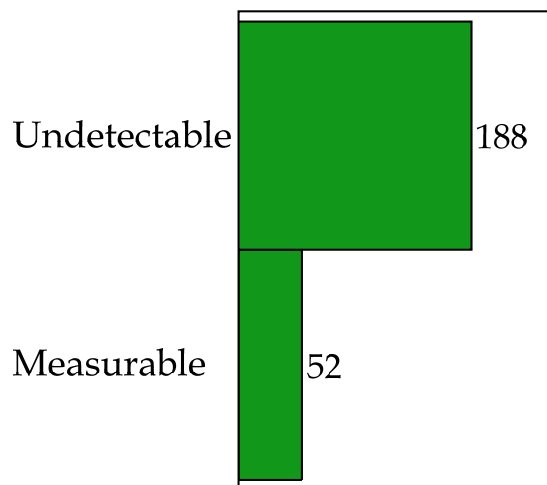


Figure 4. The concentration of RANKL measurable from the buccal side of the premolars.

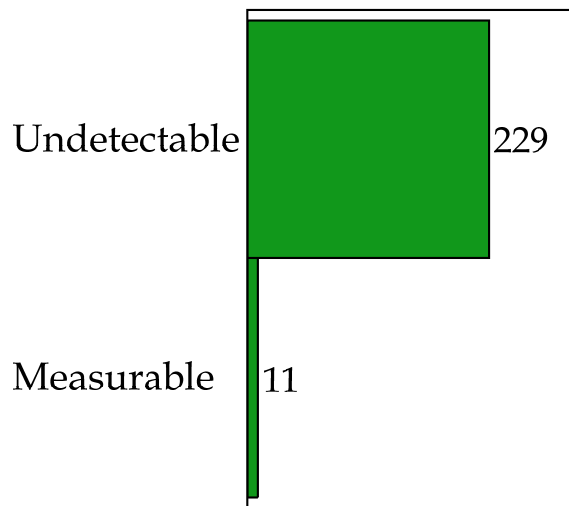


Figure 5. The concentration of OPG measurable from the lingual side of the premolars.

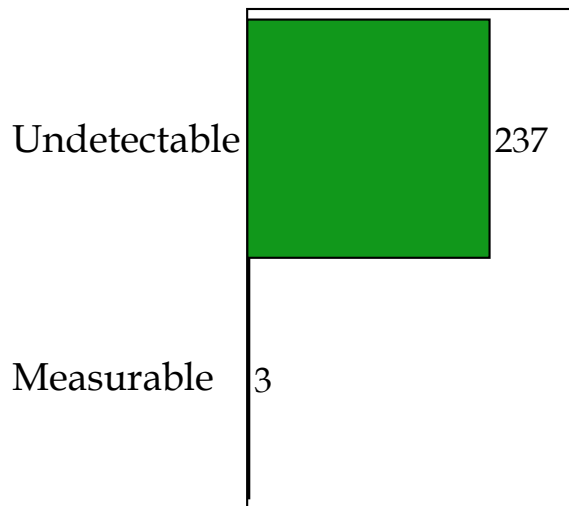


Figure 6. The concentration of OPG measurable from the buccal side of the premolars.

Figure 7 shows the plot between the volume of crevicular fluid and the concentration of RANKL (on the lingual side of premolars). This plot is most informative in that other comparisons depend on far fewer values. A second-order polynomial shows that both the linear and quadratic terms are significant statistically. The coefficient of determination is 0.299 ( $n = 52$ ;  $r = 0.55$ ), indicating that crevicular fluid volume accounts for 30% of the variation in RANKL concentration. Since the volume of fluid can be increased by more-completely absorbing the fluid during sampling, these results suggest that larger samples would enhance the number of cases with detectable values of RANKL or OPG.

### Crevicular Fluid Volume

The crevicular fluid was wicked up using one perio paper strip separately on the buccal and lingual aspects of the premolars. The volume changed significantly across the five examinations when looking at the lingual side of the tooth (Figure 8; Table 2). By Wilcoxon test, chi-square was 51.4 ( $P < 0.0001$ ), which agrees with a one-way ANOVA ( $F = 12.4$ ;  $P < 0.0001$ ). As suggested visually by the graph, the volume was low at day 0; it was higher across the next 3 examinations; and it dropped down again by day 17. Statistically, the volumes at day 0 and 17 are the same (and significantly lower than at the other 3 examinations).

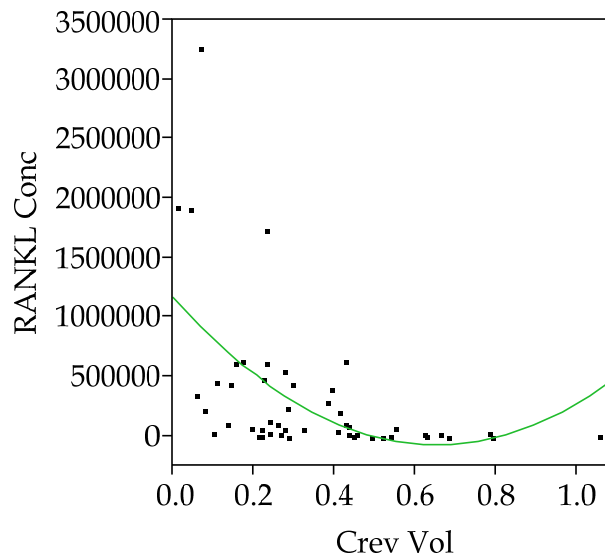


Figure 7. Bivariate plot between crevicular fluid volume and RANKL concentrations.

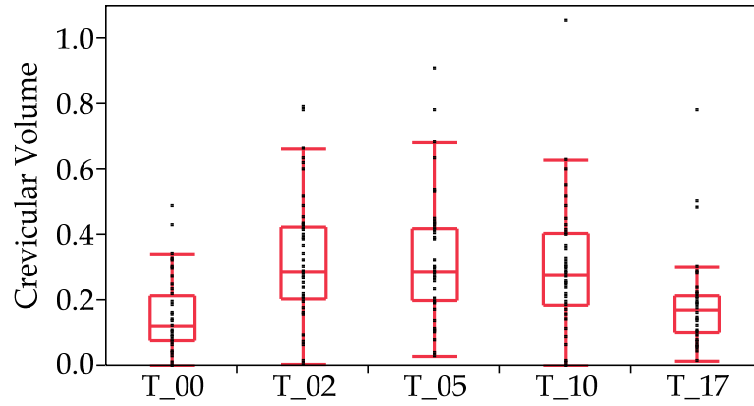


Figure 8. Box plots of the amounts of crevicular fluid obtained from the lingual side of the premolars at each of the 5 examinations.

Table 2. Descriptive statistics for crevicular fluid from the lingual side of the premolars, by examination.

Exam	n	Mean	St Error
T_00	48	0.151667	0.02352
T_02	48	0.326229	0.02352
T_05	48	0.323083	0.02352
T_10	48	0.299813	0.02352
T_17	48	0.183063	0.02352

The same pattern occurred for the buccal crevice (Figure 9; Table 3), where, again, the first and last examinations produced significantly less volume than at the intermediate 3 examinations. By Wilcoxon test, chi-square = 15.5 ( $P = 0.0038$ ), which agrees with one-way ANOVA ( $F = 4.3$ ;  $P = 0.0022$ ).

Of course, these similarities between the two sides of the tooth imply that the fluid is fairly mobile around the tooth. The volumes collected between the two sides of the tooth are positively correlated ( $r = 0.51$ ;  $P < 0.0001$ ); however, this correlation is fairly low ( $r^2 = 0.26$ ), suggesting that there also are relevant local factors influencing the volumes collected. By paired t-test (all examinations combined), there is no systematic difference in the average volumes collected between the two sides ( $t = 0.06$ ;  $P = 0.52$ ).

For completeness, we also tested for a sex difference (all examinations combined). No suggestion of a sex difference in the volume of fluid suggested. For the lingual collections,  $F = 0.5$  ( $P = 0.46$ ). For the buccal collections,  $F = 0.1$  ( $P = 0.82$ ).

### RANKL Concentrations

The concentrations of RANKL varied enormously, by several orders of magnitude, both within and among examinations. For the lingual collections, RANKL was high (in some samples) at the pretreatment examination, and then showed only trivial concentrations – and no change – across the subsequent four examinations (Figure 10; Table 4). By Wilcoxon test, the change is suggestive ( $X^2 = 8.1$ ; 4 df;  $P = 0.0881$ ), but not significant statistically.

RANKL concentrations on the buccal side of the premolars (Figure 11; Table 5) likewise exhibit no statistically significant change across time. Concentrations are trivially higher at the first 2 examinations, but median levels actually are low across all examinations. By Wilcoxon test, RANKL does not respond to the orthodontic force ( $X^2 = 3.4$ ; 4 df;  $P = 0.49$ ).

Curiously (and perhaps because of the collection protocol), there typically was more volume collected on the lingual aspect. The least-squares regression line fit to all of the data is: buccal volume =  $0.128 + 0.504$  (lingual volume). When the volumetric samples were small, more fluid was obtained from the buccal aspect of the tooth. As the volume increased, the buccal-lingual difference diminished, and at relatively high volumes, more fluid was collected from the lingual aspect (Figure 12).



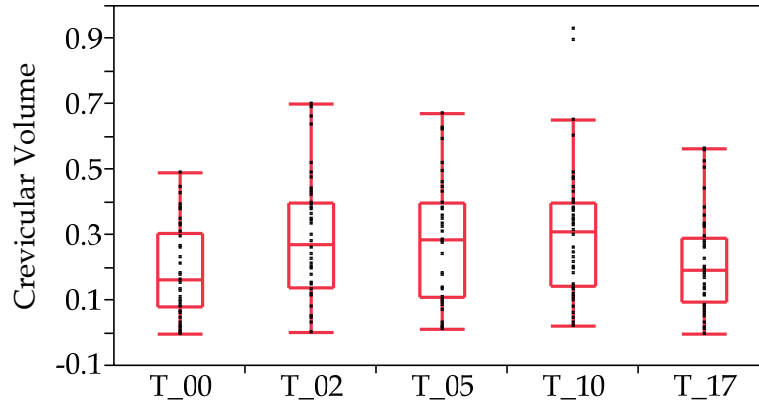


Figure 9. Box plots of the amounts of crevicular fluid obtained from the buccal side of the premolar at each of the 5 examinations.

Table 3. Descriptive statistics for crevicular fluid from the buccal side of the premolars, by examination.

Exam	n	Mean	St Error
T_00	48	0.189625	0.02480
T_02	48	0.290188	0.02480
T_05	48	0.282792	0.02480
T_10	48	0.308229	0.02480
T_17	48	0.216396	0.02480

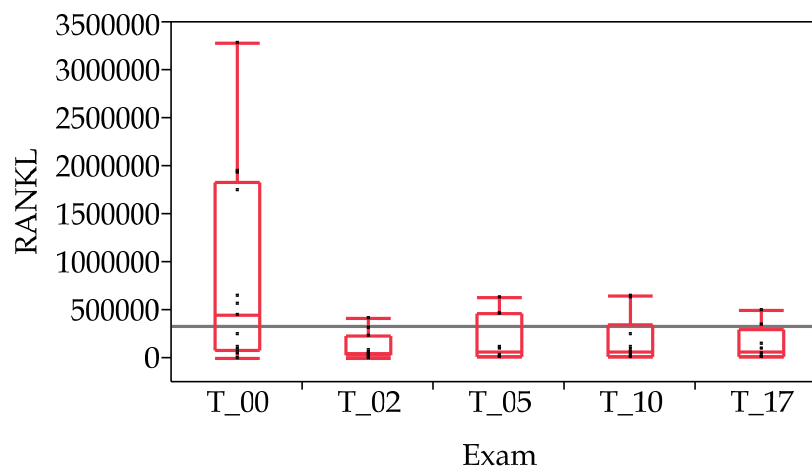


Figure 10. Box plots of the concentrations of RANKL by examination from the lingual side of the premolars.

Table 4. Descriptive statistics for RANKL from the lingual side of the premolars, by examination.

Exam	n	Mean	St Error
T_00	13	858,678	152835
T_02	11	114,373	166149
T_05	10	192,520	174259
T_10	10	190,596	174259
T_17	8	149,875	194827

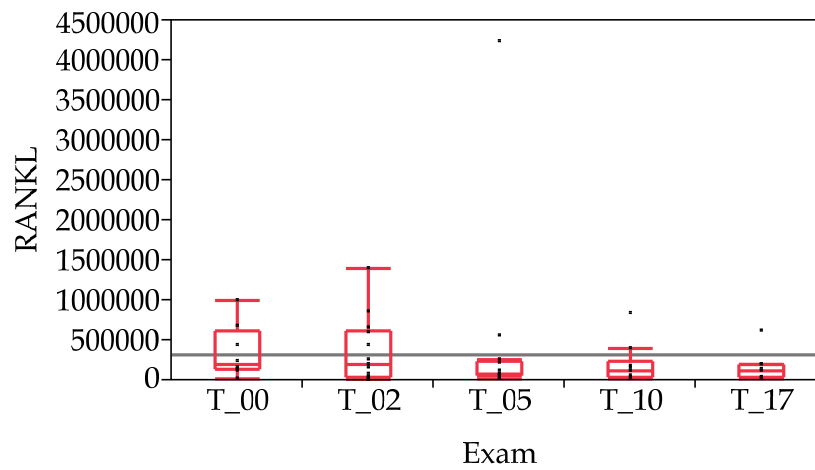


Figure 11. Box plots of the concentrations of RANKL from the buccal side of the premolars.

Table 5. Descriptive statistics for RANKL from the buccal side of the premolars, by examination.

Exam	n	Mean	St Error
T_00	8	349,312	226623
T_02	13	366,653	177778
T_05	12	477,177	185037
T_10	10	197,096	202698
T_17	9	155,820	213662

### OPG Concentrations

It was tested whether OPG concentrations changed across the 5 examinations. By Wilcoxon test, there was no difference among examinations ( $X^2 = 3.0$ ; 3 df;  $P = 0.3975$ ). The mean concentrations are graphed in Figure 13 and the descriptive statistics are listed in Table 6.

Just 3 measurable cases were available for the OPG concentrations sampled from the buccal aspect of the premolars (Figure 14; Table 7), so it is not surprising that, statistically, the Wilcoxon test showed the changes across time to be non-significant ( $X^2 = 0.4$ ; 2 df;  $P = 0.37$ ).

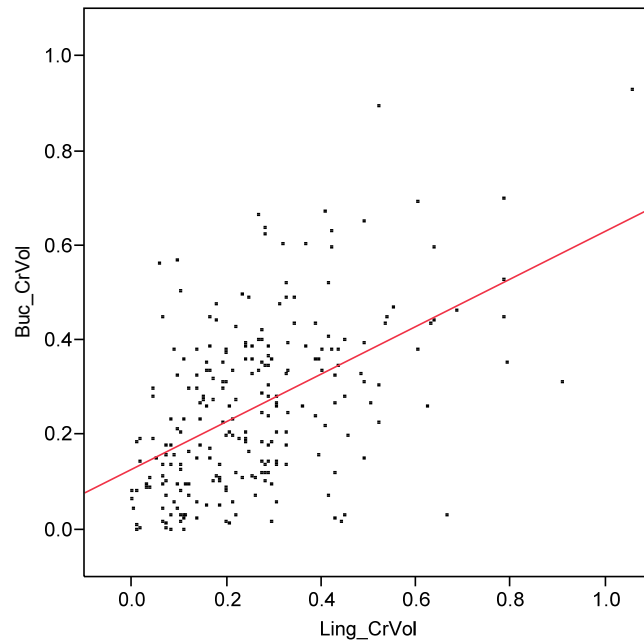


Figure 12. Bivariate plot of the relationship between the volumes of crevicular fluid obtained between the buccal and lingual aspects of the same teeth.

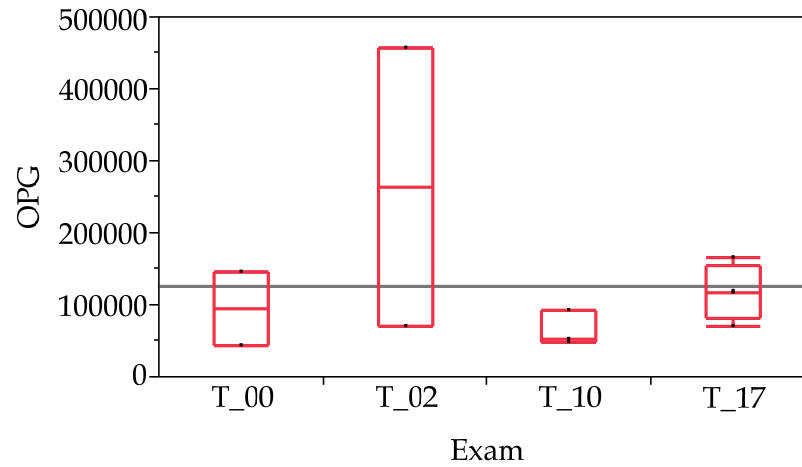


Figure 13. Box plots of the concentrations of OPG from the lingual side of the premolars.

Table 6. Descriptive statistics for the concentrations of OPG from the lingual side of the premolars, by examination.

Exam	n	Mean	St Error
T_00	2	96,127	78211
T_02	2	264,730	78211
T_10	3	65,534	63859
T_17	4	118,813	55304

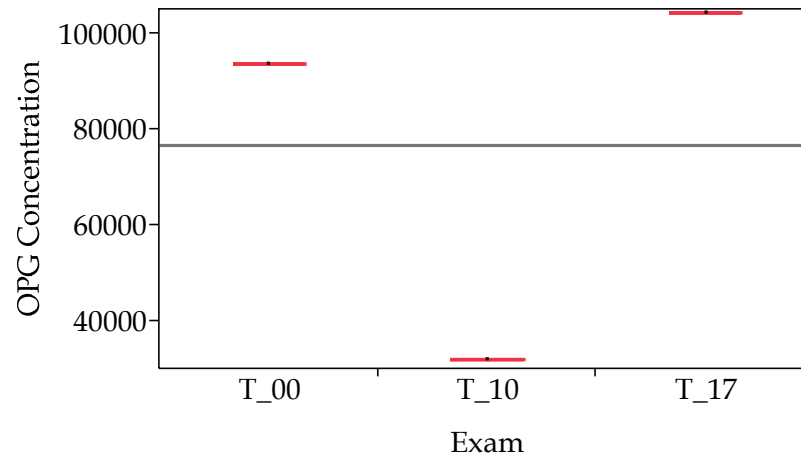


Figure 14. Box plots of the concentrations of OPG from the buccal side of the premolars.

Table 7. Descriptive statistics for the concentrations of OPG from the buccal side of the premolars, by examination.

Exam	n	Mean	St Error
T_00	1	93,750	.
T_10	1	32,124	.
T_17	1	104,497	.

## CHAPTER 5. DISCUSSION

### Gingival Crevicular Fluid Volume

The present study was designed to evaluate changes in expression of RANKL and OPG in gingival crevicular fluid (GCF) after application of orthodontic force. GCF samples were collected at five different time points over a 17 day period. A baseline sample was collected prior to force application (T\_00); three samples were collected after force initiation by a nickel titanium transpalatal spring at days 2, 5, and 10 (T\_02, T\_05, and T\_10, respectively); and the final sample was collected one week after discontinuing force delivery (T\_17).

Results show a statistically significant increase in GCF volume for both the tension and compression sides following force application. The increased levels of GCF were maintained throughout the ten days that the transpalatal spring was in place, and the levels had returned to baseline measurements by one week after spring removal. These results suggest that increased GCF volume is related to orthodontic tooth movement.

Previous reports in the literature regarding the effect of orthodontic tooth movement on GCF volume have been conflicting. Tersin (1978) observed gingival exudation during the course of orthodontic treatment and reported an increase in GCF volume. Baldwin *et al.* (1999) also reported an increase in GCF volume induced by orthodontic tooth movement. On the other hand, Miyajima *et al.* (1991) and Uematsu *et al.* (1996) reported no statistical difference between the amount of fluid expressed around teeth undergoing orthodontic movement and control teeth. A recent study investigating the change in volume of GCF exudate during canine retraction reported a slight (but nonsignificant) increase (Dannan *et al.* 2009).

A strength of the present study is the number of subjects that were enrolled (n = 48) compared to previous studies with smaller sample sizes. Due to high interindividual and intraindividual variability in GCF expression, a larger sample size is needed to detect statistically significant changes.

A somewhat interesting observation was that the elevation in GCF was maintained while the nickel-titanium spring was in place (no statistical difference between the levels collected at T\_02, T\_05, and T\_10). The tension of the spring was set in the range of 150 to 250 grams of force, which is the range suggested by Storey and Smith (1952) for optimum tooth movement. Since a nickel-titanium spring was used, constant force was delivered to the premolars over the 10 day period due to its superelastic properties (Muir *et al.* 1988). A

constant force results in uninterrupted stimulation of the cells of the periodontal ligament and alveolar bone, which produces steady bone turn-over. If another material had been used, such as an elastomeric chain, the amount of GCF produced could be expected to decrease significantly over time due to rapid force decay.

### RANKL and OPG Detectability

Gingival crevicular fluid is an exudate produced by the periodontal tissues. It is usually produced in small amounts by healthy periodontal tissues, but production increases in the presence of gingival inflammation. Research has shown that GCF production is increased in response to bacterial insult such as gingivitis (Brecx *et al.* 1987). During orthodontic tooth movement, however, only part of the increase in production of GCF can be attributed to inflammation caused by increased plaque retention (Samuels *et al.* 1993). It has also been reported that GCF production increases even when subjects undergo adequate plaque control (Tersin 1978).

The initial response to application of orthodontic force is a generalized inflammatory type reaction by the periodontal tissues (Kyrkanides *et al.* 2000). The immediate increase in GCF flow is thought to be due to mechanical compression of the microvasculature of the periodontal ligament (PDL) resulting in increased serum expression in the crevicular space. Shortly thereafter, areas of hyalinization occur due to capillary constriction constituting the initiating inflammatory event (Murrell *et al.* 1996). The release of a host of chemical mediators by the cells of the PDL in response to the areas of necrosis changes the composition of the GCF that is expressed into the gingival sulcus. This has been demonstrated by a number of studies in which expression of proinflammatory cytokines in GCF tends to increase (Uematsu *et al.* 1996; Iwasaki *et al.* 2005; Dudic *et al.* 2006; Rohaya *et al.* 2009). In the present study, however, the results show that RANKL and OPG were not consistently detectable in GCF samples before, during, or after application of orthodontic force. A likely explanation for this is that the levels of RANKL and OPG were usually below the sensitivity of the ELISA assays that were used (RANKL = 63 pg/mL; OPG = 62.5 pg/mL).

Other studies have also had problems detecting these cytokines consistently in subjects with healthy gingiva. Bostanci *et al.* (2007) conducted a study comparing GCF levels of RANKL and OPG in subjects with healthy gingiva, gingivitis, and periodontitis. Only seven of 21 healthy subjects (33%) and nine of 22 subjects with gingivitis (41%) had clinically detectable RANKL in the collected GCF. OPG was detectable in all samples from the study. However, Lu *et al.* (2006) did not detect OPG in any of the GCF samples from healthy

subjects (0 of 14 samples). Toygar *et al.* (2008) were able to detect OPG in GCF from the distal sites of canines during retraction as well as from the control teeth which were not undergoing orthodontic movement. The present study detected RANKL in 104 of 480 samples (21.6%) and OPG in 14 of 480 samples (2.92%).

Such large differences in cytokine detectability in the few studies that have analyzed GCF for RANKL and OPG could be due to varying assay sensitivity. Possible changes in protocol to overcome the limitation of assay sensitivity could include obtaining higher volumes of GCF per sample (leave the perio paper in the gingival sulcus for a longer time) or diluting the GCF samples in as little buffer as possible to obtain the highest concentrations of RANKL or OPG. Further advances in assay sensitivity will also allow detection of smaller amounts of cytokines in GCF in the future.

### Response to Periodontitis *versus* Orthodontic Force

Results from studies in the field of periodontology suggest that RANKL is more readily detectable when pathogenic inflammation is present (especially with periodontitis) compared to the aseptic inflammation observed during orthodontic tooth movement. Bostanci *et al.* (2007) reported that RANKL was detectable in all samples from subjects with periodontitis and that the levels were significantly higher than those obtained from subjects with healthy gingiva. This is understandable since the inflammation present with periodontitis and the inflammation during orthodontic tooth movement represent two different conditions of the periodontium. Although many of the same chemical mediators are present, the stimuli involved are very different as are the magnitude of the periodontal cells' response. Periodontitis is a chronic inflammatory response to bacterial insult resulting in tissue damage. The inflammation represents an immune response aimed at killing the invading bacteria. The presence of an elevated number of RANKL producing immune cells in the sulcus could explain the significantly higher levels of RANKL detected in GCF. In contrast, the condition of the periodontium during orthodontic tooth movement is different. The transmission of orthodontic force to the PDL and surrounding bone triggers a biologic response aimed at tissue remodeling characterized by selective bone resorption and deposition.

### Cytokine Expression Related to Age

Recent orthodontic research analyzing GCF and whole blood during tooth movement has found age related differences in cytokine expression. Results from Iwasaki *et al.* (2005) showed that growing individuals at an average age of



12.8 years expressed significantly more IL-1 $\beta$  in their blood and GCF than non-growing individuals with an average age of 20 years. The present study evaluated GCF for RANKL and OPG, and was limited to adolescents, age 12 to 18 years. In this restricted interval there is a miniscule elevation in RANKL across ages, but the slope is far from significant statistically ( $P = 0.47$ ). (With only 3 samples for OPG, it is meaningless to test for an age effect.) Further research is needed to determine if there is an age related difference in the expression of these two cytokines (using a broader age range), and what effect that may have on tooth movement.

### Timing of Peak RANKL and OPG Expression

As described, RANKL expression by osteoblasts is required for osteoclastogenesis and maturation of osteoclast precursor cells, and osteoblasts can also stimulate rapid bone resorption by activating pre-existing osteoclasts (Boyle, Simonet and Lacey 2003). RANKL partially regulates the survival of mature osteoclasts, in that RANKL increases their survival time. However, RANKL is not required for mature osteoclast function. After RANKL stimulates formation and activation of osteoclasts, their continued function depends on other cellular messages received by cell to cell interaction with osteoblasts. Therefore, although osteoclastic activity peaks around 48 hours after application of orthodontic force this event may not coincide with a peak in RANKL expression. The increase in RANKL expression by osteoblasts may occur much sooner after the initiation of orthodontic force.

Smith and Roberts (1980) reported a rapid acceleration of mitotic activity and DNA synthesis within two hours of application of a continuous force to rat teeth. Uematsu, Mogi and Deguchi (1996) reported an increase in GCF expression of IL-1 $\beta$ , IL-6, tumor necrosis factor (TNF)- $\alpha$ , epidermal growth factor (EGF), and  $\beta_2$ -microglobulin in as little as one hour. All of these, except  $\beta_2$ -microglobulin, peaked at 24 hours after continuous force application.

The first time point where GCF was collected in the present study was two days following force application. It is possible that large changes in the expression of RANKL and/or OPG could have occurred before the two day time point and escaped measurement. Further research using different time points is needed to determine the timing of the change in expression of RANKL and OPG after orthodontic force application.

## CHAPTER 6. SUMMARY AND CONCLUSIONS

This study evaluated changes in expression of RANKL and OPG in gingival crevicular fluid (GCF) after application of orthodontic force. The subject sample included 24 males and 24 females with an average age of 15.2 years. Of the 24 males, 18 were Caucasian and 6 were African American. Of the females, 12 were Caucasian and 12 were African American. Major findings were:

- The volume of GCF expressed into the sulcus significantly increased after application of orthodontic force and remained elevated until after removal of the force. This supports the idea that orthodontic tooth movement is associated with an inflammatory like reaction of the periodontium in response to force.
- GCF expression exhibits high inter-individual variability.
- There was no statistical difference in GCF volume between males and females or between Caucasians and African Americans at any time point.
- The levels of RANKL and OPG in the GCF collected were undetectable in the majority of the collected samples.
- Whereas RANKL and OPG are readily detectable in GCF obtained from patients with periodontitis, these cytokines appear to be expressed in smaller amounts in response to orthodontic force.
- RANKL and OPG may be present at greater levels in the tissues surrounding the teeth. The levels present in GCF may not be representative of what is occurring in the tissues during orthodontic tooth movement.
- Further research is needed using more sensitive methods to assess the changes in RANKL and OPG in response to orthodontic force, and what effect they may have on orthodontic tooth movement.

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## VITA

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